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(71) Applicant
Sandoz Ltd., 35 Lichtstrasse, CH-4002 Basle, Switzerland
(72) Inventor
Lawrence Aloysius Kelly
(74) Agents
B. A. Yorke & Co.

(54) **Sterol liposomes containing medicaments**

(57) Liposomes formed from liquid crystals of a sterol, for example cholesterol, β-sitosterol, desmosterol, 7 - keto - cholesterol, β-cholestanol or estradiol, and an aliphatic lipid capable of forming micelles in water, for example sodium or potassium salts of saturated or unsaturated C4-18 fatty acids, encapsulate medicaments (e.g. insulin, mazindol or ergot alkaloids). The medicament-containing liposomes are used by themselves or together with a pharmaceutical carrier or diluent, for oral or parenteral administration of the medicament.

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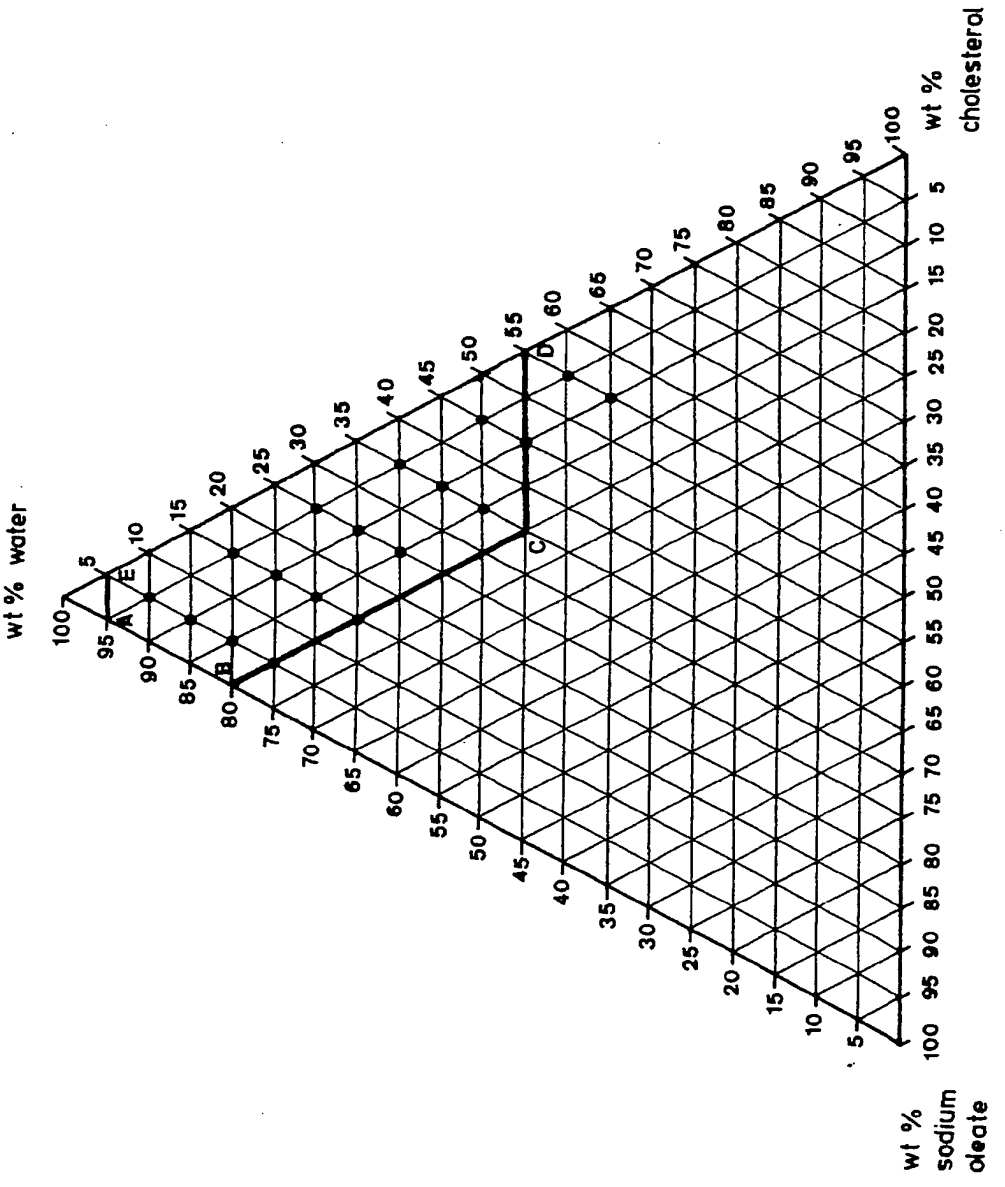
FIG. 1



SPECIFICATION

Liposome delivery systems

This invention relates to liposome delivery systems for medicaments.

Liposomes may be formed when liquid crystals of certain lipids are agitated, for example by ultrasonic radiation. They are microvesicles generally from half a micron to several microns diameter, which normally have a multilamellar structure in which the liposome walls consist of several layers of oriented lipid molecules, giving an "onion-like" structure. Larger, unilamellar liposomes may also be prepared.

It is known that medicaments may be entrapped within, or between the layers of, liposomes at the time they are formed. In all cases which have been described, the liposomes are phospholipid-cholesterol systems, which are prepared by dissolving the phospholipid and cholesterol in a solvent which is evaporated to leave a thin film of lipid. An aqueous solution of medicament is added, which swells the phospholipid/cholesterol mixture to give a liquid crystalline system. Ultrasonic treatment then gives liposomes which encapsulate the medicament.

It has now been found that liposomes capable of encapsulating drugs may be prepared without the use of complex phospholipids. In place of a phospholipid is used an aliphatic lipid capable of forming micelles in water.

Accordingly, the present invention provides a liposome medicament delivery system in which a medicament is encapsulated within liposomes comprising a sterol and an aliphatic lipid capable of forming micelles in water.

The aliphatic lipid may be any pharmacologically acceptable aliphatic surface-active compound which forms micelles in aqueous media when present in concentrations above the critical micelle concentration (CMC). A micelle is a colloidal-sized aggregation of surfactant molecules in an aqueous medium, in which the molecules are oriented with their hydrophilic ends outwards and their lipophilic ends inwards. Preferably the aliphatic lipid is a sodium or potassium of a C_4 to C_{18} saturated or unsaturated fatty acid. Suitable acids include butyric, isovaleric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic acids. The salt is more preferably the salt of an unsaturated fatty acid, particularly of 14 to 18 carbon atoms. Particularly preferred are sodium and potassium oleate.

The sterol may be any pharmacologically acceptable sterol capable of forming liposomes with the above aliphatic lipids. Preferred sterols are cholesterol, β-sitosterol, demosterol, 7-keto-cholesterol, β-cholestanol and estradiol, particularly cholesterol and β-sitosterol.

The nature of the medicament to be encapsulated is not critical. Suitable medicaments include vaccines and antigens, as well as drugs. Suitable drugs include hormones, e.g. insulin; ergot alkaloids, e.g. dihydroergotoxin, dihydroergotamine and bromocryptine and anoretics, e.g. mazindol.

The invention also provides a process for the preparation of the liposomal delivery system, in which either

a) solid crystals of the sterol are contacted with an aqueous micellar solution of the aliphatic lipid and the medicament, and the resulting liquid crystals are converted to liposomes by ultrasonic irradiation,

b) the sterol is dissolved in a mixture of an aqueous medium containing the aliphatic lipid and medicament with a water-miscible solvent, and the solvent is evaporated,

or c) the sterol and medicament are dissolved in a common solvent, the solvent is evaporated, the residue is contacted with an aqueous micellar solution of the aliphatic lipid and the resulting liquid crystals are converted to liposomes by ultrasonic irradiation.

In process variant a), the sterol crystals are contacted with the micellar solution at a temperaure of up to 60°C, preferably 20°-50°C, more preferably 25°-45°C. Penetration of the sterol crystals by the micelles requires from 2-60 minutes, and sonication should be carried out only after penetration is complete and a liquid crystal system has been formed.

In process variant b), the water-miscible solvent is suitably acetone, dioxane, or a C_{1-4} alcohol. The sterol is dissolved with stirring at a temperature of up to 60°C, preferably 20-50°C. Evaporation of the water-miscible solvent reduces the liquid crystals to liposomes without ultrasonic irradiation being required.

Process variant c) is suitable where the medicament to be encapsulated is not soluble in the aqueous micellar solution of the aliphatic lipid. A suitable common solvent for the sterol and the medicament may for example be chloroform, benzene or petroleum ether. The residue after evaporations is contacted with the aqueous micellar solution and subjected to ultrasonic irradiation under the same preferred conditions as in variant a).

In all three process variants, the process is preferably carried out under an inert atmosphere, for example nitrogen, to prevent autooxidation of the lipid and/or the sterol.

The proportions of aliphatic lipid, sterol and water in the liposome delivery systems of the invention may be: aliphatic lipid 0.03%-20%, sterol 1.0%-55%, water 45%-97%. Preferably the proportions are: aliphatic lipid 1.0%-15%, sterol 1%-40%, water 50%-97%; more preferably aliphatic lipid 5%-10%, sterol 1.0%-10%, water 75%-95% (by weight).

In general, suitable proportions are those in which liquid crystals are seen to be formed on contacting the sterol with a micellar solution of the aliphatic lipid in water. For the system sodium oleate/cholesterol/water, these proportions have been determined experimentally by adding aqueous solutions of sodium oleate to solid cholesterol in the proportions shown in Table I, equilibrating for 48 hours and examining for the presence of liquid crystals.

Table I

Sample No.	% oleate	% cholesterol	% water	liq. crystals formed?
1	5	5	90	yes
2	5	15	80	yes
3	5	25	70	yes
4	5	35	60	yes
5	5	45	50	yes
6	5	55	40	no
7	10	5	85	yes
8	10	15	75	yes
9	10	25	65	yes
10	10	35	55	yes
11	10	45	45	yes
12	10	55	35	no
13	15	5	80	yes
14	15	15	70	yes
15	15	25	60	yes
16	15	35	50	yes
17	20	15	65	yes
18	20	5	75	yes

Figure 1 shows a phase diagram of the three-component system sodium oleate/cholesterol/water. Liquid crystalline phases were found within the area ABCDE on the phase diagram. Similar phase diagrams may be constructed for other systems according to the invention.

The composition of the total delivery system is not of course the same as that of the liposomes themselves. Thus the liposomes will contain essentially all the sterol and much of the aliphatic lipid present in the system, but will be suspended in a continuous aqueous phase, which may also contain micelles of the aliphatic lipid, or molecules of the aliphatic lipid at concentrations below the CMC.

The liposomes prepared according to the invention are from 10 to 600 nm in diameter.

The liposome delivery system of the invention may be used both for oral and for parenteral administration of medicaments, optionally after concentration or isolation of the liposomes for example by ultracentrifugation. Oral administration is preferred, however, as the liposome encapsulation may serve to protect drugs such as insulin which are labile in the digestive system. For oral administration the liposome suspension may be admixed with pharmacologically acceptable diluents or carriers and with conventional adjuvants such as flavourings and colourings, and administered in such forms as syrups, elixirs, capsules etc. For parenteral administration, the concentrated or isolated liposomes may be suspended in a suitable carrier liquid, for example sterile distilled water or physiological saline solution. Suppositorial administration may also be utilised.

The following Examples illustrate the invention:

EXAMPLE 1:

Two concentrations of bovine insulin were prepared:

1) 2500 international units (IU) or 102.9 mg (Specific Activity = 24.3 units/mg) was dissolved in 5 ml of a 16 g/l aqueous sodium oleate solution,
2) 1250 international units or 51.45 mg (Specific Activity = 24.3 units/mg) was also dissolved in 5 ml of a 16 g/l aqueous sodium oleate solution.

Each of insulin preparations (1) and (2) were transferred to a 10 ml beaker containing 20 mg of cholesterol. The cholesterol was prepared by dissolving 200 mg of cholesterol in 10 ml of chloroform, then placing one ml of the solution in a 10 ml beaker and removing the solvent under nitrogen at room temperature. Each beaker was flushed with nitrogen, covered with plastic film and placed in a 37°C water bath with mild oscillation for 1 hour to allow penetration of the cholesterol crystals. The liquid crystal suspensions were then sonicated for 2 one minute periods with a Biosonik IV Ultrasonic Generator (Brownwill) with a 4 mm diameter probe. The beakers were placed in an ice bucket during the sonication. After sonication, the final compositions of the liposome-containing systems were:

Composition A)	insulin	= 20.9 mg/ml
	cholesterol	= 4 mg/ml
	sodium oleate	= 16 mg/ml
Composition B)	insulin	= 10.4 mg/ml
	cholesterol	= 4 mg/ml
	sodium oleate	= 16 mg/ml.

Suspensions of the liposome compositions A) and B) were given orally to mice, 0.1 ml of the suspension per 10 g body weight being administered. This corresponds to 5000 IU/kg of composition A) or 2500 IU/kg of composition B).

Composition A) was diluted 1:10 for injection i.m. and administered at 10 IU/kg, as was bovine pancreatic insulin (Sigma Chemical).

After two and four hours the animals were sacrificed by anethetizing with 85 mg/kg of sodium hexobarbital i.p. and collecting blood via cardiac puncture. The collected blood was placed in an Auto

Analyzer Cup containing 0.025 ml of heparin, 1000 units/ml. The blood samples were capped, shaken, and kept in an ice bucket. Glucose was determined by the Auto Analyzer potassium ferricyanide method No. N-2b.

Carboxymethyl cellulose has no effect on blood glucose whether given either by the p.o. or the i.m. route. Therefore, the test materials whether given p.o. or i.m. can be related to the carboxymethyl cellulose control.

Normal fasting blood glucose levels are from 70 to 100 mg/100 ml.

The results are shown in Table II.

Table II

BLOOD GLUCOSE LEVELS (mg/100 ml)					
Treatment	Route	2 Hours Post	% Δ	4 Hours Post	% Δ
Control Carboxymethyl cellulose	p.o.	79.8 ± 6.4	—	103.0 ± 3.6	—
A) 5000 IU/kg	p.o.	72.5 ± 8.5	9 ↓ P=N.S.	75.3 ± 9.0	27 ↓ P=<0.5
B) 2500 IU/kg	p.o.	68.8 ± 4.0	14 ↓ P=N.S.	96.0 ± 10.5	6 ↓ P=N.S.
A) 10 IU/kg	i.m.	23.8 ± 2.3	70 ↓ P=<0.001	28.3 ± 3.6	73 ↓ P=<0.001
Bovine 2 Pancreatic Insulin 10 IU/kg	i.m.	14.0 ± 0.8	82 ↓ P=<0.001	26.3 ± 5.8	75 ↓ P=<0.001

P = probability that results could be obtained by chance
NS = not statistically significant

Significant reduction of the blood glucose level was seen 4 hours after oral dosing with 5000 IU/kg insulin in the liposomes of this invention. In the i.m. route, liposome-encapsulated insulin had equivalent activity to unencapsulated insulin at 10 IU/kg, indicating that the insulin activity was not effected by incorporation into the liposomes.

The liposomes of composition A) were also compared with both positively and negatively charged lecithin-cholesterol liposomes, prepared as described in Weissmann, G., et al., Proc. Nat. Acad. Sci. USA 72.88-92 (1975); Sessa, G. & Weissmann, G., J. Biol. Chem. 245. 3295-3301 (1970); Weissmann, G., Brand, A. & Franklin, E.C., J. Clin. Invest. 53. 536-543 (1974); and Weissmann, G. & Rita, G.A., Nature 240. 167-172 (1972), in the presence of 500 IU/ml of bovine insulin.

The liposomes of this invention and the lecithin liposomes were both tested in mice and analyzed as described above.

The results ars shown in Table III.

Table III

Treatment	Route	BLOOD GLUCOSE (MG/100 ML) 2 Hours Post	% Δ	4 Hours Post	% Δ
Control Carboxymethyl cellulose	p.o.	112.0 ± 5.9	—	92.7 ± 6.7	—
A) 5000 IU/kg	p.o.	47.6 ± 10.3	58 ↓ P=<0.01	70.2 ± 8.7	24 ↓ P=N.S.
L.C. (+) 5000 IU/kg	p.o.	105.8 ± 17.8	17 ↓ P=N.S.	92.8 ± 5.8	0 P=N.S.
L.C. (−) 5000 IU/kg	p.o.	88.9 ± 7.6	21 ↓ P=<0.05	91.5 ± 12.5	1 ↓ P=N.S.
A) 5 IU/kg	i.m.	24.8 ± 2.5	78 ↓ P=<0.001	21.0 ± 2.7	77 ↓ P=<0.001
L.C. (+) 5 IU/kg	i.m.	31.8 ± 2.6	72 ↓ P=<0.001	24.3 ± 3.1	74 ↓ P=<0.001
L.C. (−) 5 IU/kg	i.m.	29.0 ± 3.5	74 ↓ P=<0.001	18.3 ± 1.6	80 ↓ P=<0.001

The results show that at both time periods the liposomes of this invention were superior to the lecithin phospholipid liposomes when given p.o. All preparations were equally effective when given intramuscularly.

EXAMPLE 2:

Liposomes were prepared by dissolving 20 mg (25.3 IU/mg) of bovine insulin in a solution of 0.5 g sodium oleate in 9 g of water, and then adding 0.5 cholesterol crystals. The composition was allowed to equilibrate and was sonicated as described in Example 1.

The liposomes were isolated by ultrafiltration in a centrifuge rotated at 20,000 RPM for 2 hours. A 40% sucrose underlayer was used to fill the tube.

Three fractions were isolated;

1) a clear top layer,
2) the liposome layer, and
3) an infranatent above the sucrose layer.

The three fractions were tested by administration to mice at a dose of 0.1 ml of each fraction/10 g of body weight. The animals weighed from 20-30 g and were fasted overnight prior to testing.

Each fraction had a separate control:

1st fraction — insulin in water (20 mg insulin/9 ml water);

2nd fraction — liposomes previously prepared without insulin, to which insulin was added prior to adminstration to the mice (20 mg insulin/9 ml liposome mixture);

3rd fraction — 20 mg insulin/9 ml water and included the sucrose cushion to allow for the effect on blood glucose.

The animals were dosed orally as described in Example 1 and the results analyzed as described in Example 1.

The results were as follows:

	BLOOD GLUCOSE MG/100 ML Control	Fraction	%
Top Fraction	152 ± 6	206 ± 2	35
Liposomes	164 ± 8	136 ± 14	17
Infranatant	151 ± 16	173 ± 20	15

Only the liposome entrapped insulin decreases blood glucose. The other fractions tend to elevate blood glucose.

EXAMPLE 3:

A slurry of 7.5 g cholesterol in 100 ml of 99% pure acetone is poured into a 2 1 beaker and evaporated so that the cholesterol is dispersed evenly over the bottom of the beaker. A 5% aqueous micellar solution of sodium oleate is prepared by dissolving 10 g purified sodium oleate in 200 ml distilled water, and 400 mg dihydroergotoxine methanesulphate is dissolved in this solution. 150 ml of this sodium oleate / dihydroergotoxine solution is added to the cholesterol, and the mixture is stirred under nitrogen at room temperature for 1 hour, then ultracentrifuged at 20,000 rpm for 24 hours.

Three fractions were isolated: a) an upper clear yellow layer (40% vol) containing micelles of sodium oleate, b) a middle viscous yellow-white layer (20% vol) and c) a lower opaque white lower layer (20% vol) containing liposomes.

Spectrophotometric assay for dihydroergotoxin was carried out by the van Urk method, in which equal quantities of the dihydroergotoxin-containing

solution and the van Urk reagent are mixed, left to stand for 30 minutes, filtered and the absorbance of the solution at 550 nm compared with that of a mixture of a standard dihydroergotoxin solution and the van Urk reagent. The van Urk reagent is prepared by dissolving 2.5 g p-dimethylaminobenzaldehyde in a mixture of 700 ml distilled water with 1300 ml conc. sulphuric acid, adding 4 ml of 5% aqueous ferric chloride solution, and making up to 2 litres with distilled water.

The results showed no variation in dihydroergotoxin concentration in the free fractions, indicating that at least some of the dihydroergotoxin had become encapsulated in the liposomes.

CLAIMS

1. A liposome medicament delivery system in which a medicament is encapsulated within liposomes comprising a sterol and an aliphatic lipid capable of forming micelles in water.

2. A delivery system as claimed in Claim 1 in which the sterol is cholesterol, β-sitosterol, desmosterol, 7-keto-cholesterol, β-cholestanol or estradiol.

3. A delivery system as claimed in Claim 2 in which the sterol is cholesterol or β-sitosterol.

4. A delivery system as claimed in any one of the preceding claims in which the aliphatic lipid is a sodium or potassium salt of a C_4 to C_{18} saturated or unsaturated fatty acid.

5. A delivery system as claimed in Claim 4 in which the aliphatic lipid is a sodium or potassium salt of a C_{14} to C_{18} unsaturated fatty acid.

5. A delivery system as claimed in Claim 1 in which the sterol is cholesterol and the aliphatic lipid is sodium or potassium oleate.

7. A delivery system as claimed in any one of the preceding claims in which the medicament is insulin.

8. A delivery system as claimed in any one of the preceding claims in which the medicament is an ergot alkaloid.

9. A delivery system as claimed in any one of the preceding claims, in which the proportions by weight of aliphatic lipid, sterol and water are 0.03%-20%, 1.0%-55% and 45%-97% respectively.

10. A delivery system as claimed in Claim 9 in which the proportions by weight of aliphatic lipid, sterol and water are 1.0%-15%, 1.0%-40% and 50%-97% respectively.

11. A delivery system as claimed in Claim 10 in which the proportions by weight of aliphatic lipid, sterol and water are 5%-10%, 1.0%-10% and 75%-95% respectively.

12. A delivery system as claimed in Claim 8 in which the aliphatic lipid is sodium oleate and the proportions by weight of sodium oleate, cholesterol and water are such as lie within the area ABCDE on the triangular graph of Figure 1.

13. A process for the preparation of a liposome medicament delivery system as claimed in Claim 1 in which either

a) solid crystals of the sterol are contacted with an aqueous micellar solution of the aliphatic lipid and the medicament, and the resulting liquid crystals are converted to liposomes by ultrasonic irradiation,

b) the sterol is dissolved in a mixture of an aqueous medium containing the aliphatic lipid and medicament with a water-miscible solvent, and the solvent is evaporated,

or c) the sterol and medicament are dissolved in a common solvent, the solvent is evaporated, the residue is contacted with an aqueous micellar solution of the aliphatic lipid and the resulting liquid crystals are converted to liposomes by ultrasonic irradiation.

14. A process as claimed in Claim 13, carried out at 20°-50°C in an inert gas atmosphere.

15. A process for the preparation of a liposome medicament delivery system substantially as described in any one of Examples 1-3.

16. A liposome medicament delivery system whenever prepared by the process of any one of Claims 13-15.

17. A pharmaceutical composition comprising a liposome medicament delivery system as claimed in any one of Claims 1 to 12 and 16, in admixture with a pharmaceutically acceptable diluent or carrier.

18. A pharmaceutical composition as claimed in Claim 17 for parenteral administration, in which the liposomes are suspended in sterile distilled water or physiological saline solution.

19. A pharmaceutical composition as claimed in Claim 17, for oral administration, in the form of a syrup, elixir or capsule.

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